Supplemental Figures

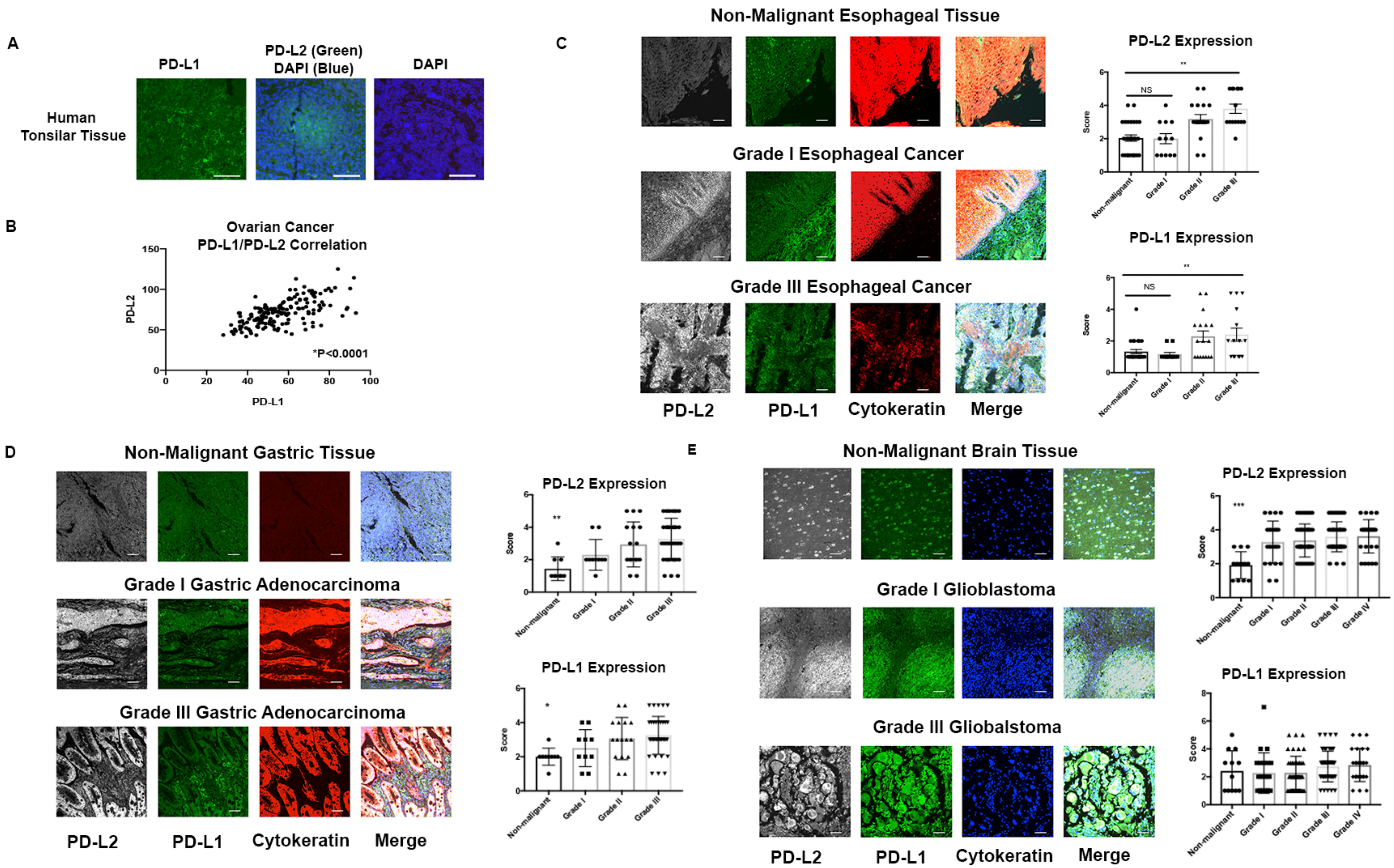
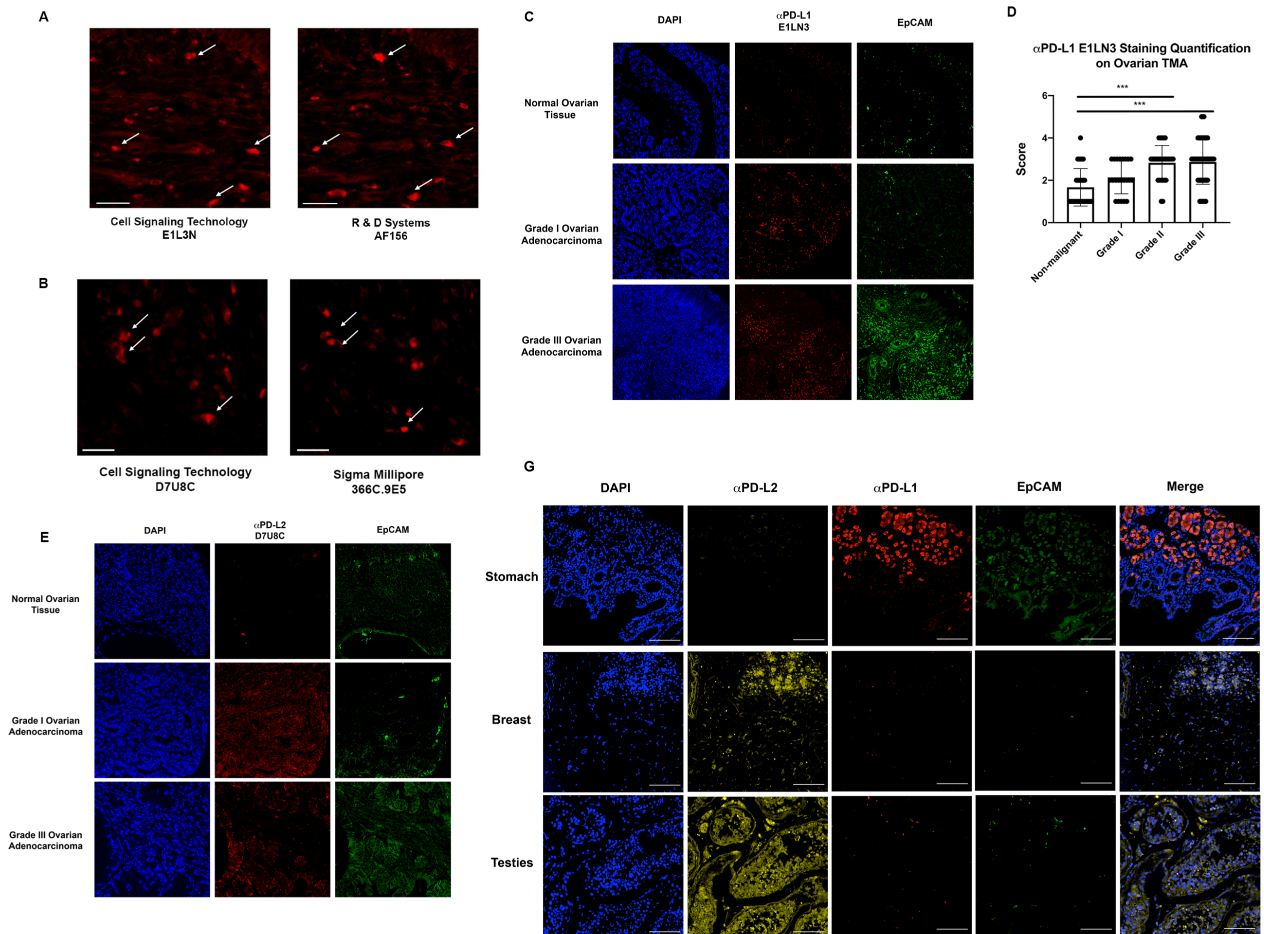
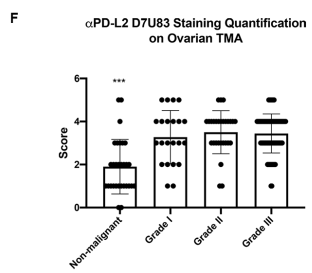


Fig. S1. PD-L1 and PD-L2 expression analyzed in gastric, esophageal and brain cancer. (A) IF staining of human PD-L1 and PD-L2 on tonsil tissue showing distinct staining pattern for PD-L1 and PD-L2 expression. Scale bar 100μM. (B) Pearson correlation plot of PD-L1 and PD-L2 expression in ovarian cancer. (C) Representative images of esophageal cancer tissue microarray containing both normal and malignant samples (N=72) were stained with anti-human PD-L2 (gray), anti-human PD-L1 (green) and DAPI (blue) by fluorescent immunohistochemistry. The intensity of PD-L2 (right upper) and PD-L1 (right bottom) was scored and stratified according to the tumor grade. (D) Representative images of gastric cancer tissue microarray containing both normal and malignant samples (N=76) were stained with anti-human PD-L2 (gray), anti-human PD-L1 (green) and DAPI (blue) by fluorescent immunohistochemistry. The intensity of PD-L2 (right upper) and PD-L1 (right bottom) was scored and stratified according to the tumor grade. (E) Representative images of glioblastoma tissue microarray containing both normal and malignant samples (N=152) were stained with anti-human PD-L2 (gray), anti-human PD-L1 (green) and DAPI (blue) by fluorescent immunohistochemistry. The intensity of PD-L2 (right upper) and PD-L1 (right bottom) was scored and stratified according to the tumor grade. Scale bar 50μM.

A





H

Organ type	PD-L1	PD-L2	Co-localize with EpCam
Lymph node	-	+++	NO
Thymus	-	+++	YES
Testies	+	+++	NO
Breast	-	++	NO
Small Intestine	+	+	NO
Cervix	-	-	NO
Skin	+	-	NO
Bone	+	-	NO
Spleen	+++	-	NO
Prostate	++	-	NO
Pancreas	+	-	Partial
Rectal	+	-	NO
Esophageal	++	-	NO
Lung	+	-	YES
Stomach	+++	-	YES
Colon	-	-	NO
Liver	++	-	Partial

Fig. S2. PD-L1 and PD-L2 expression in human tumor and normal tissue. (A) Expression pattern of PD-L1 detected in same ovarian tumor tissue using two clones of aPD-L1 antibody. E1LN3 from Cell Signaling Technology (CST) on the left panel and AF156 from R & D systems on the right panel. (B) Expression pattern of PD-L2 detected in same ovarian tumor tissue using two clones of aPD-L2 antibody. D7U8C from Cell Signaling Technology on the left panel and 366C.9E5 from Sigma Millipore on the right panel. (C) Representative images of ovarian cancer TMA stained with PD-L1 (CST E1L3N) and EpCAM. (D) The intensity of PD-L1 staining was scored and stratified according to the tumor grade. (E) Representative images of ovarian cancer TMA stained with aPD-L2 (CST D7U8C) and EpCAM. (F) The intensity of PD-L2 staining was scored and stratified according to the tumor grade. (G) Representative images of normal tissues from multiple organs were stained with aPD-L1, aPD-L2 and EpCAM. (H) Staining intensity of PD-L1, PD-L2 as well as spatial expression co-localizing with EpCAM is analyzed and tabulated according to tissue site.

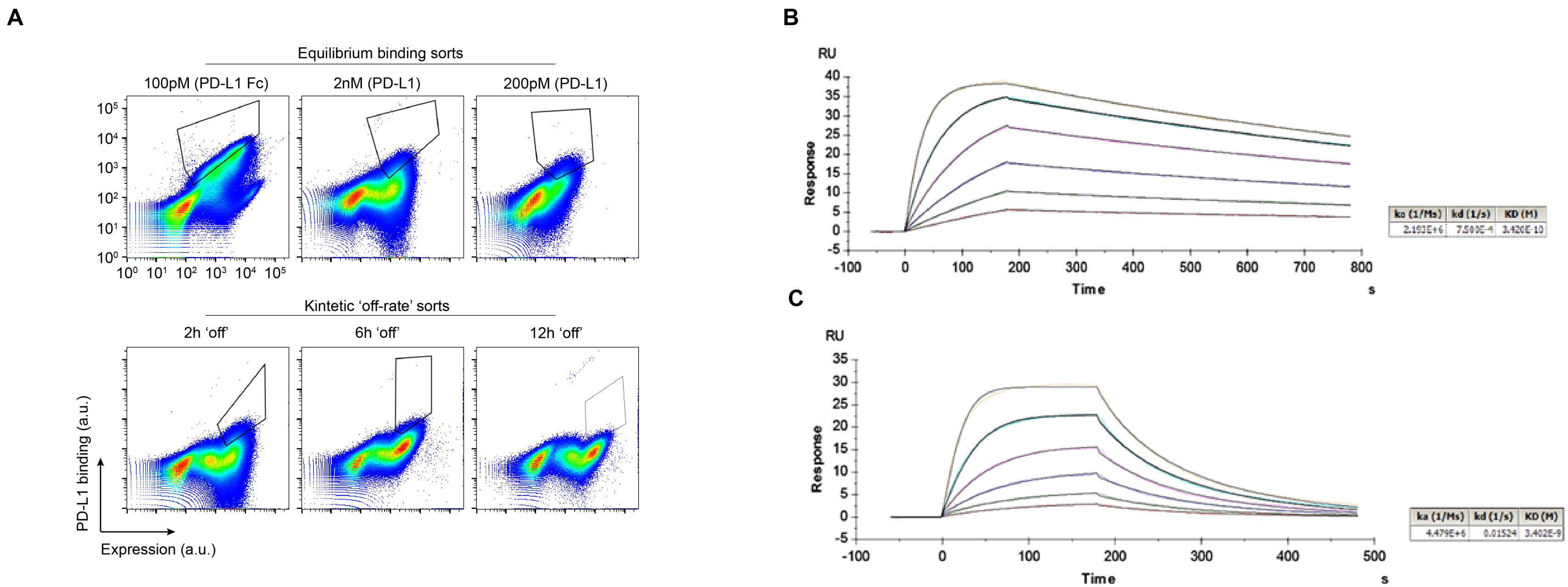
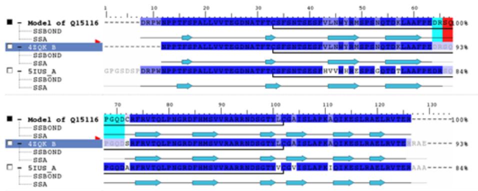


Fig. S3. Flow cytometry plots showing gating strategy for the shuffled library and SPR traces of mutant sPD-1 clones exhibiting improved binding. A) The first three rounds were performed as equilibrium binding sorts with stringency being increased by lowering the concentration of PD-L1 used to label the library. Note that the first sort was performed with hPD-L1 Fc and subsequent sorts used hPD-L1 to enable interrogation of a monomeric interaction. The final three sorts employed kinetic 'off-rate' sorts to further impart selective pressure and enable selection of only the strongest binding clones. Gates used to collect the top 1 – 3% of the library are shown. Binding analysis of sPD-1V2 binding to kinetics to PD-L1 (B) and PD-L2 (C) calculated by BIAcore T200 at 25 °C. Each curve represents the binding curve of the analyte at a single dose concentration.

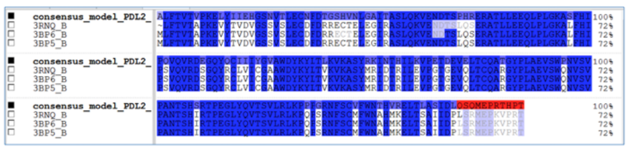
A



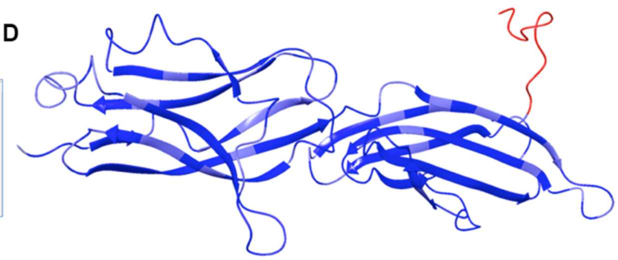
B

Site in PD-1	Surface Complementarity		Hydrogen Bond with PD-L1	
	WT	Single mutation	WT	Single mutation
G124S	0.93	0.75	0	1 to Y123
S127V	0.89	0.64	0	0
A132I	0.72	0.85	1 to Q66	2 to Q66

C



D



E

Site in PD1	Surface Complementarity		Hydrogen bond with PD-L2	
	WT	Single mutation	WT	Single mutation
G124S	0.80	0.85	0	0
A132I	0.83	0.86	1 to Q60	1 to Q60

Fig. S4. Sequence alignment and protein interaction between sPD-1 mutants, PD-L1 and PD-L2. (A) Sequence alignment of modeled wild-type PD-1 (Q15116) with PDB No. 4ZQK and 5IUS. The identical residues are marked in blue, the residues missing in both 4ZQK and 5IUS are highlighted in red and the residues missing only in 4ZQK are marked in cyan. (B) Analysis of surface complementarity and hydrogen bond for each of the three mutations within the binding interface on sPD-1 mutants when in complex with human PD-L1. (C) Sequence alignment between simulated model of human PD-L2 superimposed with three mouse PD-L2 structures from PDB (PDB No. 3RNQ, 3BP6, 3BP5). The residues in missing loop are marked red. (D) Consensus model of human PD-L1. (E) Analysis of surface complementarity and hydrogen bond for 2 mutations within the binding interface on sPD-1 mutants in co-complex with hPD-L2.

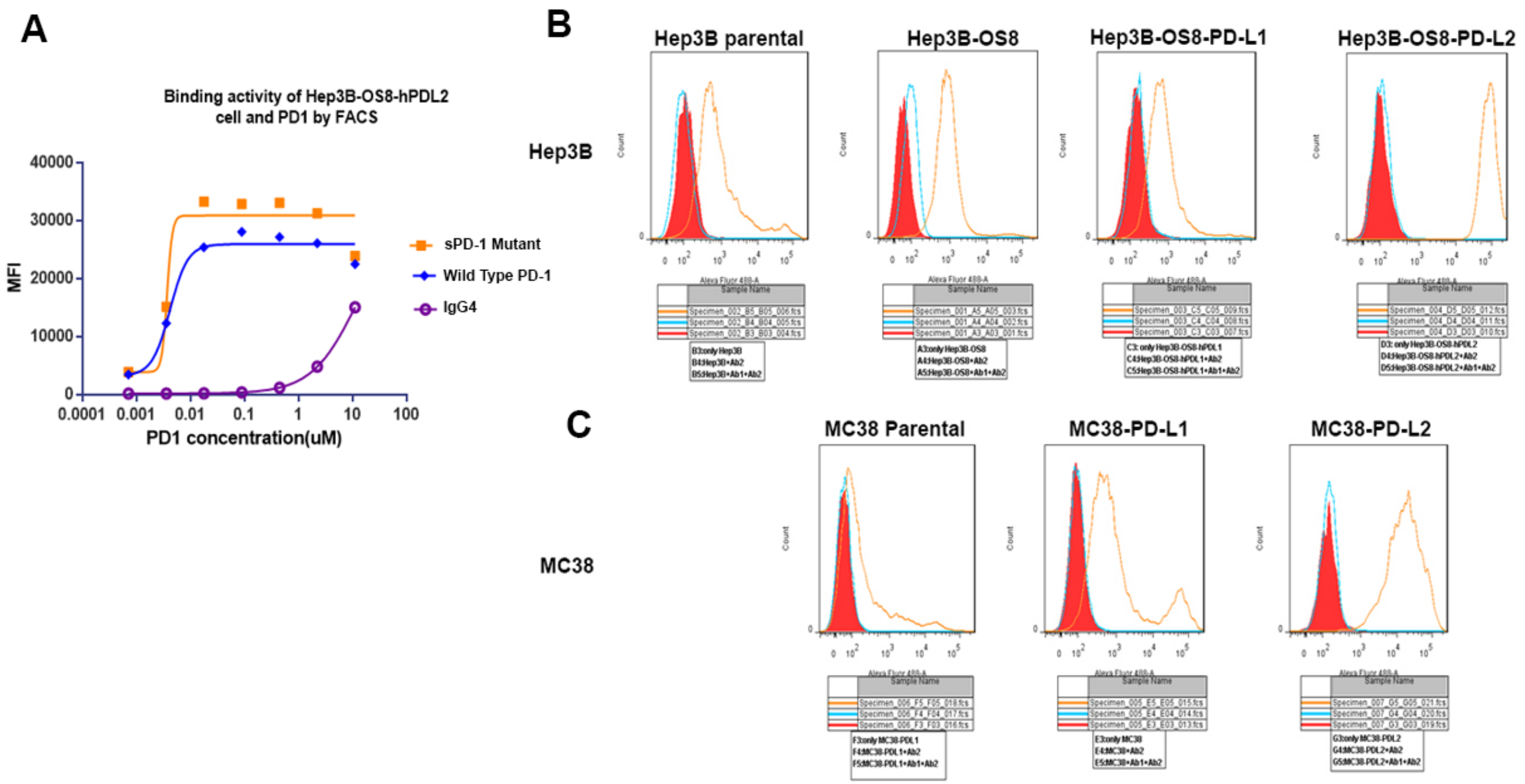


Fig. S5. sPD-1 binding to human PD-L2. (A) Binding kinetics between sPD-1V2 binding to PD-L2 expressing Hep3B-OS8-PDL2 cells. Each data point was the average value of a technical duplicate. (B) Validation of human PD-L2 expression on Hep3B-OS8 and MC38 cells. (C-F) Positive clones were sorted from the lowest efficiency to highest efficiency on the right. Ab1 is anti-human PD-L2 antibody and Ab2 is control IgG.

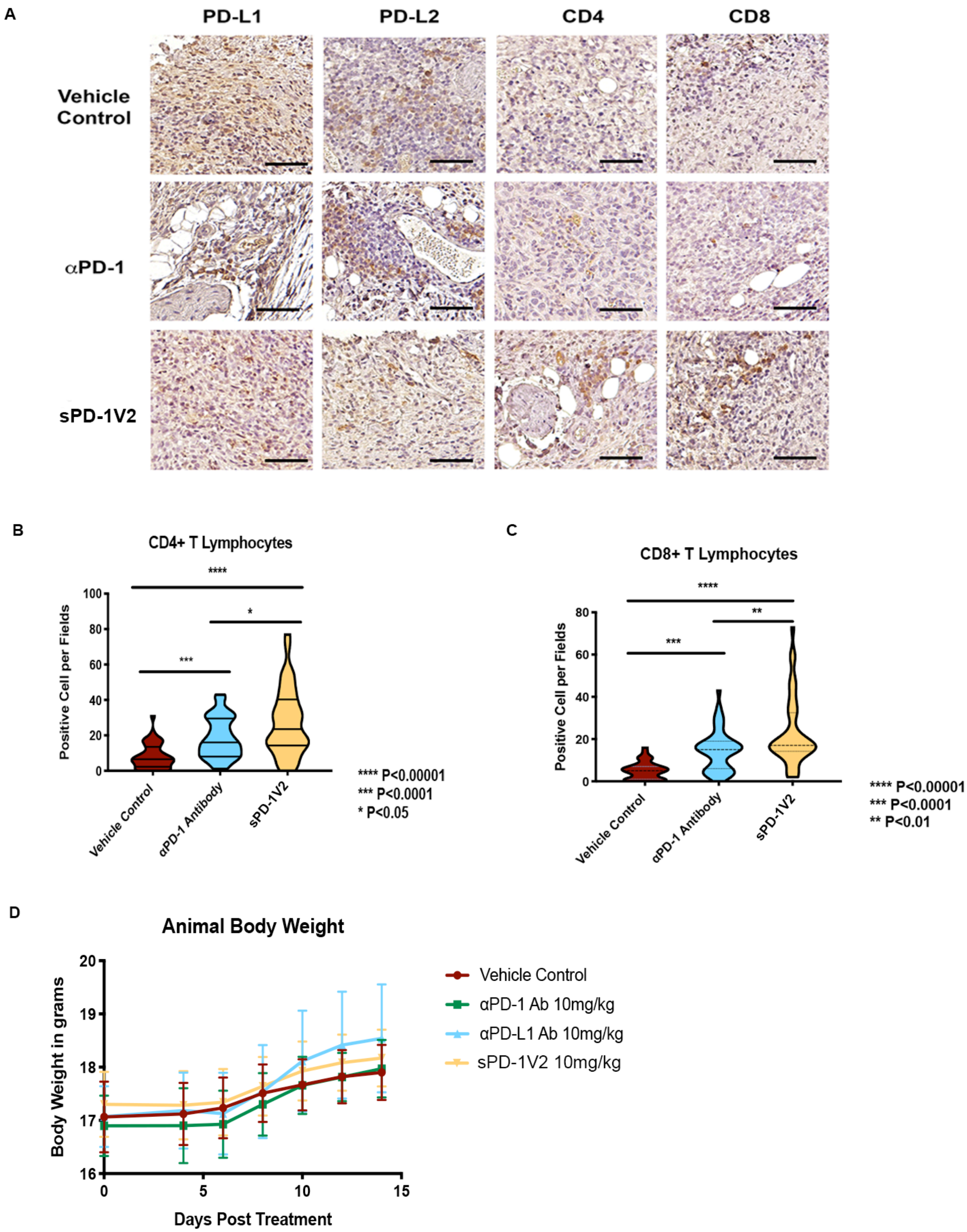

A
PD-L1
PD-L2
CD4
CD8
Vehicle Control
αPD-1
sPD-1V2
B
CD4+ T Lymphocytes
Positive Cell per Fields
Vehicle Control
αPD-1 Antibody
sPD-1V2
**** P<0.00001
*** P<0.0001
* P<0.05
C
CD8+ T Lymphocytes
Positive Cell per Fields
Vehicle Control
αPD-1 Antibody
sPD-1V2
**** P<0.00001
*** P<0.0001
** P<0.01
D
Animal Body Weight
Body Weight in grams
Days Post Treatment
Vehicle Control
αPD-1 Ab 10mg/kg
αPD-L1 Ab 10mg/kg
sPD-1V2 10mg/kg

Fig. S6. sPD-1V2 treatment leads to increased CD4+ and CD8+ TIL infiltration. (A) The expression of PD-L1, PD-L2, CD4 and CD8 positive cells in ID8 tumors post-treatment was analyzed by immunohistochemical staining. Scale bar 30μM. (B) Violin quantitative plot of CD4+ T lymphocyte infiltrated into ID8 ovarian tumors post treatment. (C) Violin quantitative plot of CD8+ T lymphocyte infiltrated into ID8 ovarian tumors post treatment. (D) Mice body weight overtime during the UPK10 ovarian cancer study. Statistical analysis was conducted using One-way ANOVA for comparing between treatment groups and repeated ANOVA for changes occur over-time. P value *=<0.05, **=<0.01. ***=<0.001.

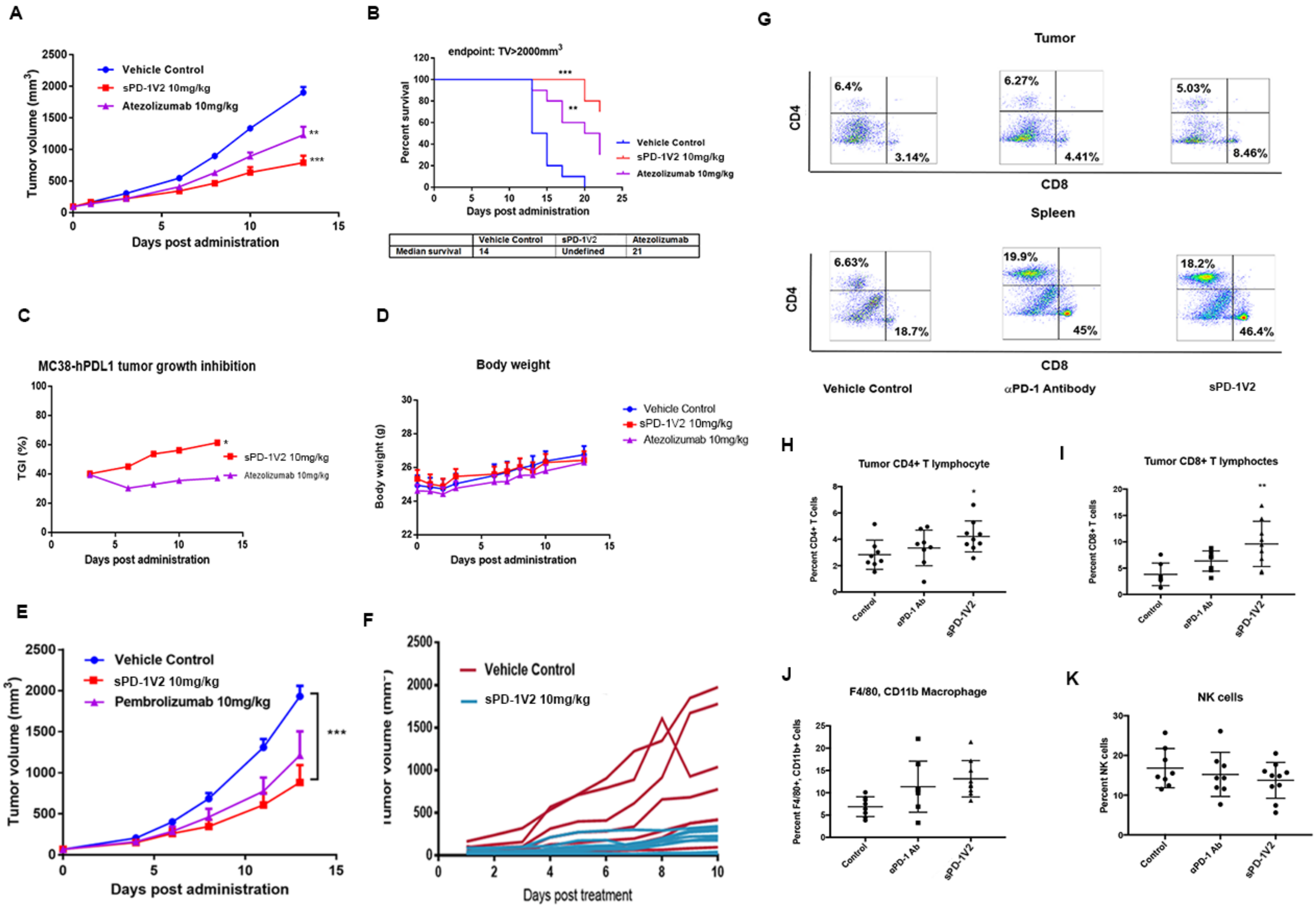

A
Vehicle Control
sPD-1V2 10mg/kg
Atezolizumab 10mg/kg
Tumor volume (mm3)
Days post administration
B
endpoint: TV>2000mm3
Percent survival
Vehicle Control
sPD-1V2 10mg/kg
Atezolizumab 10mg/kg
Days post administration
| | Vehicle Control | sPD-1V2 | Atezolizumab |
| Median survival | 14 | Undefined | 21 |
C
MC38-hPDL1 tumor growth inhibition
TGI (%)
sPD-1V2 10mg/kg
Atezolizumab 10mg/kg
Days post administration
D
Body weight
Body weight (g)
Vehicle Control
sPD-1V2 10mg/kg
Atezolizumab 10mg/kg
Days post administration
E
Vehicle Control
sPD-1V2 10mg/kg
Pembrolizumab 10mg/kg
Tumor volume (mm3)
Days post administration
F
Vehicle Control
sPD-1V2 10mg/kg
Days post treatment
G
Tumor
CD4
6.4%
3.14%
6.27%
4.41%
5.03%
8.46%
CD8
Spleen
CD4
6.63%
18.7%
19.9%
45%
18.2%
46.4%
CD8
Vehicle Control
αPD-1 Antibody
sPD-1V2
H
Tumor CD4+ T lymphocyte
Percent CD4+ T Cells
Control
αPD-1 Ab
sPD-1V2
I
Tumor CD8+ T lymphoctes
Percent CD8+ T cells
Control
αPD-1 Ab
sPD-1V2
J
F4/80, CD11b Macrophage
Percent F4/80+, CD11b+ Cells
Control
αPD-1 Ab
sPD-1V2
K
NK cells
Percent NK cells
Control
αPD-1 Ab
sPD-1V2

Fig. S7. sPD-1V2 treatment demonstrates superior anti-tumor activity in colorectal cancer compared to αPD-1 antibody (A) C57B/6 mice inoculated with the MC38-hPD-L1 colorectal cancer then assigned to vehicle control, sPD-1V2 10mg/kg or Atezolizumab 10mg/kg. (N=10) (B) Kaplan Meier survival plot showing animals been terminated upon reaching ethical endpoint for tumor growth with median survival listed below. (N=10) (C) Percent of tumor growth inhibition comparing sPD-1V2 and Atezolizumab treated groups. (D) Total body weight of mice treated with vehicle control, sPD-1V2 and Atezolizumab during the experiment. N=10 for each treatment group. Error bar represents mean and standard deviation. (E) Tumor growth over time in C57B/6 mice inoculated with MC38-hPD-L2 colorectal cancer then assigned to vehicle control, sPD-1V2 10mg/kg or Pembrolizumab10mg/kg. Each data point represents mean and SEM of individual tumor measured over-time. (F) Tumor growth over time in C57B/6 mice inoculated with B16/OVA melanoma cells then treated with to vehicle controls or sPD-1V2 10mg/kg. (N=8) (G) Representative flow cytometry dot plots of $CD4^+$ and $CD8^+$ cytotoxic T-cells isolated from tumor (upper panel) and spleen (lower panel) of B16/OVA melanoma tumors treated with vehicle control, anti-mouse αPD-1 blocking antibody 10mg/kg and sPD-1V2 10mg/kg. (H) $CD4^+$ and (I) $CD8^+$ T cells isolated and analyzed from MC38 tumors treated with vehicle control, anti-mouse αPD-1 blocking antibody 10mg/kg and sPD-1V2 10mg/kg. (J) Percent positive NK cells in the tumors of MC38 tumors treated with vehicle control (N=8), anti-mouse αPD-1 antibody (N=8) and sPD-1V2 antibody (N=10). Individual data point, mean and standard deviation shown. (K) Percent positive macrophages in the tumors of MC38 tumors treated with vehicle control (N=8), anti-mouse αPD-1 antibody (N=8) and sPD-1V2 (N=10). Individual data point, mean and standard deviation are shown. Statistical analysis was conducted using One-way ANOVA for comparing between treatment groups and repeated ANOVA for changes occur over time. P value *=<0.05, **=<0.01. ***=<0.001.

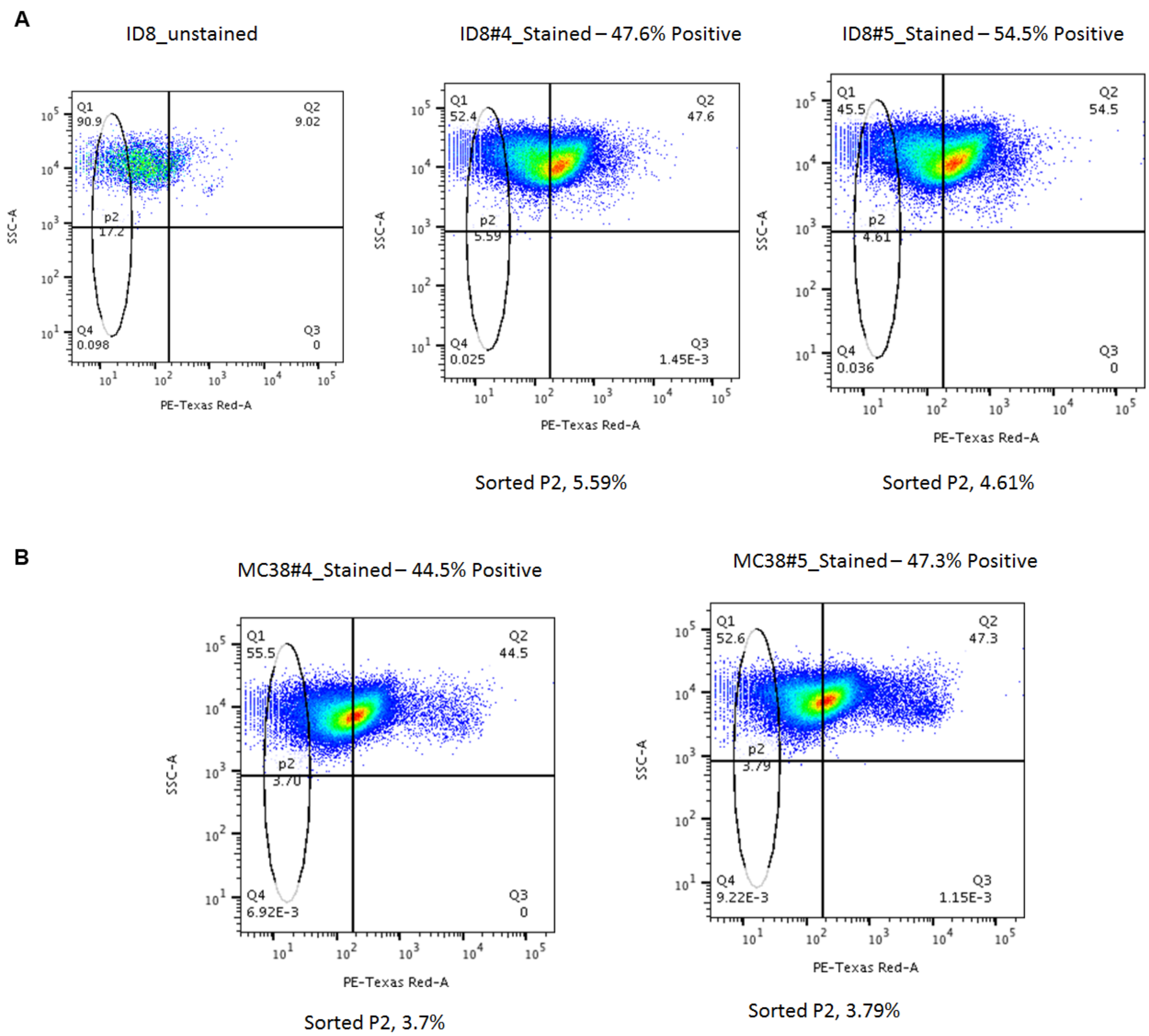


Fig. S8. Isolation of PD-L1 negative cells post PD-L1 CRISPR transfection. (A) PD-L1 negative ID8 cells sorted and collected post PD-L1 CRISPR clone 4 (left) and clone 5 (right) transfection. (B) PD-L1 negative MC38 cells sorted and collected post PD-L1 CRISPR clone 4 (left) and clone 5 (right) transfection.

Supplemental Tables

Table S1. Sequencing of enriched sPD-1 library pool after five rounds of sorting the error-prone library. The residue number is listed at the top and the corresponding wild-type amino acid is given. Frequently occurring mutations are highlighted.

Error-prone Library - sort 5 sequences																																
	33	37	45	49	50	59	70	74	76	87	88	89	91	93	98	99	102	107	112	115	116	124	125	127	131	132	135	139	140	145	147	148
wt PD1	N	F	T	N	A	T	M	N	T	S	Q	P	Q	S	T	Q	N	H	R	R	N	G	A	S	K	A	K	R	A	T	R	R
S5.1 a																										V						
S5.1 c										G					I											V						
S5.1 e																					S					V				I		
S5.1 f							V				R					R			G		S					V						
S5.1 g						A		D		G		L														I						
S5.1 i																		R			S	S				V						
S5.1 j																										V			V			
S5.1 k	D																				D					I						
S5.1 n																				G	D					V			V			
S5.1 o																										V			V			
S5.1 p	D																							L	R	I	R	G			K	G
S5.1 q												L														V	R		V			
S5.1 t				S					A										G		S	S				V	R			A		
S5.2 b																										V			V			
S5.2 c						A		D		G		L														I						
S5.2 d								D			R								G							V	R					
S5.2 f		L																			D					V						
S5.2 g																			G							I						
S5.2 h																										V						
S5.2 i			A											R			S							F	R	I						
S5.2 l												L														V	R		V			
S5.2 m																										V			V			
S5.2 o								S																		V						
S5.2 q	S	L			V					G			R													V						
S5.2 s								S													S		V			V				I		

Table S2. Sequencing of enriched sPD-1 library pool after six rounds of sorting the shuffled library. The residue number is listed at the top and the corresponding wild-type amino acid is given. Consensus mutations are highlighted.

Shuffled Library - sort 6 sequences																									
	49	50	57	70	77	87	89	91	92	96	99	107	110	113	116	124	127	131	132	135	139	140	145	147	148
wt PD-1	N	A	S	M	D	S	P	Q	D	R	Q	H	V	A	N	G	S	K	A	K	R	A	T	R	R
S6.8.1	S					G	L					R			S	S	L	R	I	R	G				
S6.8.2						G					R		M		S	S			I			V			
S6.8.3						G	L		A						S	S	L	R	I	R		V			
S6.8.6		V				G	L		G	G					S	S	L		I	R	G			K	G
S6.8.9	S					G	L								S	S	L	R	I			V	A		
S6.8.10			N			G	L				R				T	S	L		I			V			
S6.8.12						G			G	G	R					S	L		I		G		I		
S6.8.13						G	L								S	S	V		I			V			
S6.12.1			N			G	L				R				T	S	L		I			V			
S6.12.2		V				G	L		G	G					S	S	L		I	R	G			K	G
S6.12.3						G	L							V	S	S	L	R	I						
S6.12.4						G	L		G	G	R				S	S			V			V			
S6.12.5						G		R				R			S	S	L		I		G			K	G
S6.12.6				I		G	L								S	S	L	R	I	R	G	V		K	
S6.12.7						G	L								S	S	V		I			V			
S6.12.8					N	G			G	G	R					S	L		V			V			
S6.12.9						G	L								S	S	V	R	I			V			
S6.12.13	S					G	L					R			S	S	L	R	I	R	G				

Supplemental Materials and Methods

Synthesis of yeast-displayed sPD-1 library

DNA encoding the human PD-1 extracellular domain was cloned into a modified pCT yeast display plasmid using *NheI* and *BamHI* restriction sites. A point mutation (C93S) was made in the wild-type gene to remove a natively occurring unpaired cysteine to avoid any covalent associations on the yeast cell surface, and the display plasmid was rearranged such that PD-1 was fused at its C-terminus to Aga2p, leaving a free N-terminus. An error-prone library was created using the PD-1 extracellular domain DNA as a template and mutations were introduced by using low-fidelity Taq polymerase (Invitrogen, CA) and the nucleotide analogs 8-oxo-dGTP and dPTP (TriLink Biotech, CA). Six separate PCR reactions were performed in which the concentration of analogs and the number of cycles were varied to obtain a range of mutation frequencies: 8 and 12 cycles (200 μM of analogs), and 12 and 20 cycles (2, 20 μM of analogs). Products from these reactions were gel purified to remove template plasmid DNA and amplified using forward and reverse primers each with 50 bp homology to the pCT plasmid in the absence of nucleotide analogs. Amplified DNA was purified using gel electrophoresis, and pCT plasmid was digested with *NheI* and *BamHI*. Purified insert DNA and linearized plasmid were electroporated in a 3:1 ratio by weight into freshly made EBY100 yeast where they were assembled *in vivo* through homologous recombination[40]. Library size was estimated to be 3.3×10^8 by dilution plating.

The shuffled library was created by mixing DNA recovered from the final round of sorting with DNA encoding wild-type PD-1 with the A132V concensus mutation in a 4:1 ratio. Tris-HCl and $MgCl_2$ were added to 7.5 μgrams of pooled DNA to a final concentration of 50mM and 10mM, respectively. After mixing with 1 unit of DNase I (Thermo Scientific), digestion was allowed to proceed for 3 minutes at 15°C after which it was held at 90°C for 15 minutes to stop the reaction. This reaction was directly used as template for a self-assembly PCR reaction, without amplifying primers. Self-assembled, full-length genes were subsequently amplified in a second PCR reaction using forward and reverse primers each with 50 bp homology to the pCT plasmid. The resulting library was transformed into yeast as previously described, yielding a library of approximately 2.6×10^8 transformats as estimated by dilution plating.

Library screening

Yeast displaying high-affinity PD-1 mutants were isolated from the libraries using fluorescence-activated cell sorting (FACS). For the initial error-prone library, five rounds of equilibrium binding sorts were done using the following concentrations of PD-L1 Fc (R&D Systems, MN): round 1, 250nM; round 2, 20nM; round 3, 1nM; round 4, 0.1nM; round 5, 0.1nM. For these sorts, yeast were incubated at room temperature in phosphate buffered saline with 0.1% BSA (PBSA) with the appropriate concentration of PD-L1 Fc for 3 hours. After primary incubation with PD-L1 Fc, yeast were pelleted, washed, and resuspended in PBSA with 1:250 of chicken anti-c-Myc (Invitrogen, CA) for 1 h at 4 °C. Yeast were then washed, pelleted and secondary labeling was performed on ice for 30 min using PBSA with a 1:100 dilution of goat anti-chicken Alexa Fluor 555 (Invitrogen, CA) and goat anti-human 488 (Invitrogen, CA). Labeled yeast were sorted by FACS using a Vantage SE flow cytometer (Stanford FACS Core Facility) and CellQuest software (BD Biosciences, CA). Sorts were conducted such that the 1–3% of clones with the highest PD-L1 binding/c-Myc expression ratio were selected, enriching the library for clones with the highest binding affinity to PD-L1 Fc. In sort 1, 10^8 cells were screened and subsequent rounds analyzed a minimum of ten-fold the number of clones collected in the prior sort round to ensure adequate sampling of the library diversity. Selected clones were propagated and subjected to further rounds of FACS. Following sort 5, plasmid DNA was recovered using a Zymoprep kit (Zymo Research Corp. CA), transformed into XL-1 blue supercompetent cells, and isolated using plasmid miniprep kit (Qiagen, MD). Sequencing was performed by Sequetech Corp.

Sorting of the shuffled library was done as decribed with the following modifications. For sort 1, 100pM of PD-L1 Fc was used to label the library. Subsequent sort rounds were done using monovalent human PD-L1 with a C-terminal hexahistadine tag was used in place of PD-L1 Fc. To detect binding of the his-tagged ligand, a mouse anti-his Hilyte Fluor 488 antibody (Anaspec, CA) was used. Sorts 2 and 3 were equilibrium sorts using 2nM and 0.2nM PD-L1, respectively. Following sort 3, stringency was increased by switching to kinetic off-rate sorts. For these sorts, the library was incubated with a saturating amount of PD-L1 (5nM) for 3 hours at room temperature. Following primary incubation, yeast were pelleted, washed to remove unbound PD-L1 and resuspended in PBSA containing 50nM PD-L1 Fc as competitor to render any unbinding event permanent. The off-rate step was 2 hours for sort 4, 6 hours for sort 5, and 12 hours for sort 6.

Analysis of yeast-displayed sort products was performed using the same reagents and protocols and described for the library sorts. Samples were analyzed on a FACSCalibur (BD Biosciences, CA) and data was analyzed using FlowJo software (Treestar Inc.).

Computational modeling analysis

Human PD1/PDL1 complex structure is available in Protein Data Bank, the PDBID is 4ZQK and 5IUS. However, in both of the PDB structures, some residues of PD1 are missing in a loop area, and some of the residues were mutated compared to wild-type PD1. The missing loop and mutated residue were reconstructed back to the wild-type PD1 based on crystal structure 4ZQK. The mutated PD1 structure was modeled using the Residue Scanning module in the Schrodinger Suite. The residues within 5.0 Å of the mutated residue were refined with side-chain prediction and backbone sampling. The calculation of variation of binding affinity and complex stability were also performed for multiple mutations. Negative values are indicative of better binding affinity and stability. The homology model of PD-L2 was built to investigate the interaction between human PD1 and PD-L2. After homology search in PDB non-redundant data set, three PDB structures of mouse PD-L2 were chosen as templates.

Surface Plasmon Resonance

Human PD-L1 (#10084-H08H, SinoBiological, PA) and human PD-L2 (#10292-H08H, SinoBiological, PA) were used as analyte. Running buffer HBS-EP+ (10mM HEPES, 150mM NaCl, 3mM EDTA and 0.05% P20, pH 7.4) was prepared. Flow rate was performed at 30 µL/min. PD1-Fc was captured to the immobilized anti-human Fc IgG sensorchip. An injection of serial diluted analyte was used. Glycine pH 1.5 for 30s was used as the surface regeneration. Multiple cycle kinetics was carried out on Biacore T200. Kd analysis was calculated using the Biacore T200 Evaluation Software (GE Healthcare Life Sciences, IL).

Expression of PD-L2 in Lentiviral Transduced Hep3B-OS8 Cells and MC38 Cells

Hep3B (OS8) and MC38 cell lines were transduced with pLVX-IRES-hygro-hPDL2 (Chempartner, Shanghai, PRC). Cells were harvested according to standard procedures and the supernatant was discarded. Cells were dispensed into a round bottom 96-well staining plate with $3x10^5$ cells per well. The plate was centrifuged at 300g at 4°C for 5 minutes. 100µL of rabbit anti-hPD-L2 in titrated 1xPBS+2%FBS FACS buffer was added to each well and incubated for one hour at 4°C. Cells were washed twice with 200µL FACS buffer and centrifuged at 300g for 5 minutes. Supernatant was discarded before and after each wash. Cells were re-suspended at 100μL/well with 1:1000 dilution of secondary donkey-anti-rabbit IgG(H+L) Alexa488 life technology antibody (Invitrogen, CA) and incubated for 1 hour at 4°C. Cells were again washed twice with 200μL FACS buffer and centrifuged at 300g for 5 minutes. Supernatant was discarded before and after each wash. Cells were re-suspended in 100μL PBS. Cells were kept in the dark and submitted for FACS analysis.

Immunoassays

Slides were de-paraffined and antigen retrieval carried out using 10mM Citric Acid Buffer, 0.05% Tween 20, pH 6. Quenching endogenous peroxidase was performed with 3.4% hydrogen peroxide. Avidin and Biotin blocker were added for 15 minutes each. Protein block using 2% fetal calf serum was added for 20 minutes. The serum and antibodies were diluted in PBT (1XPBS, 0.1% BSA, 0.2% , 0.01% Tween 20), anti-Human PD-L1 1:500 (#AF154, R&D systems, MN), anti-Human PD-L2 clone 336C.9E5, 1:500 (#MABC1102, EMD Millipore, MA), anti-Human CD8 1:500 (#Ab4005, Abcam, MA), anti-Mouse PD-L1 1:750 (#17952-1-AP, Proteintech, IL), anti-Mouse PD-L2 1:750 (#Ab21107, Abcam, MA) and anti-Mouse CD8 1:500 (#Ab203035, Abcam, MA). Antibodies were incubated overnight at 4 °C. For immunohistochemistry, anti-rabbit and anti-rat were added on each slide and incubated at 37°C for 30 minutes, then incubated with STREP-HRP for 30 minutes at 37°C, and signals developed using DAB substrate kit (#34002, ThermoFisher Scientific, MA). For immunofluorescence detection, Secondary antibodies were diluted 1:400 in PBS with 2% BSA and 0.1% Tween 20 and incubated at room temperature for 1 hour. Sudan Black B was added to the slides for 10 minutes at room temperature to reduce auto-immunofluorescence. DAPI (#F6057, Sigma-Aldrich, MO) 0.1μg/mL was used for counterstain and coverslip was applied. ELISA quantification of human IgG4 (#BMS2059, ThermoFisher Scientific, MA) was carried out per manufacturer's instruction.

Flow Cytometry Based Binding Analysis

The MC38-hPD-L1, MC38-hPD-L2, Hep3B -hPD-L1, Hep3B -hPD-L2 and parental MC38 cells were cultured in standard tissue culture conditions. Cells were harvested, the supernatant discarded, then the cells dispensed onto a staining plate at $3x10^5$ cells per well. The plate was centrifuged at 300g at 4°C for 5 minutes. Various concentrations of sPD-1 mutants and negative control were diluted in FACS buffer containing 2% FBS, 100µL/well was added. Cells were incubated for 1 hour at 4°C and washed twice with 200µL FACS buffer and centrifuged at 300g for 5 minutes. The supernatant was discarded before and after each wash. Cells were re-suspended at 100µL/well with 1:1000 diluent with anti-human IgG-Alexa 488 (#A28175, ThermoFisher, MA). Plates were incubated for 1 hour at 4°C. Cells were washed twice with FACS buffer and centrifuged at 300g for 5 minutes. Supernatant was discarded and cells were re-suspended in 100µL cold PBS. The cells were kept in the dark and FACS analysis carried out on FACS CantoII, (BD Biosciences, CA).

Receptor Blocking Assay

Hep3B-hPD-L1, Hep3B-hPD-L2, MC38-hPD-L1 and MC38-hPD-L2 cells were cultured in PRMI 1640 medium, 10% FBS, G418 and hygro, in T175 flask to a confluency of 60-80%. The cells were harvested and re-suspended into dilution buffer and then dispensed into round bottom 96-well plate Corning #3799 at the density of $2x10^5$ cells/well. sPD-1 mutant at multiple concentration: 600, 120, 24, 4.8, 0.96, 0.192, 0.00384 nM and 0 nM were re-suspended in dilution buffer. sPD-1 wild-type were also prepared in dilution buffer. The plate was centrifuged at 500g for 3 minutes and supernatants discarded. Cells were re-suspended with 50µL antibody followed by adding 50µL of ligand. The plates were incubated at 4°C for 2 hours followed by centrifugation and washed. Streptavidin-Alexa Flour 488 (#S11223, Thermo Fisher, MA) diluted 1:1000 in dilution buffer was prepared and incubated with cells at 4°C for 1 hour. The plate was centrifuged and washed then re-suspended in 200µL FACS buffer. Cells ran on FACS Canto II (BD Biosciences, CA). Curves with MFI values were drawn and IC50s and tops were calculated.

T Cell Stimulation and T proliferation Assay

Blood sample from healthy donors were diluted by an equal volume of sterile PBS and mixed by gentle shaking. 15mL of Ficoll-Paque PLUS (10-1440-02, GE Healthcare, Pittsburgh, PA) medium was transferred into a fresh 50mL centrifuge tube with a Ficoll and blood volume ration of 3:4. The diluted blood sample was then carefully layered onto the surface of the Ficoll medium to avoid mixing. The tube was centrifuged at 400 g for 30 minutes at 20°C with the max acceleration and min deceleration settings during the centrifugation. Four interfaces were observed after the centrifugation with layers of plasma, mononuclear cells, Ficoll medium, and RBCs seen from top to bottom. The layer of mononuclear cells were carefully transferred into a new sterile centrifuge tube. Sterile PBS buffer was added into the collected PBMCs for washing. The tube was centrifuged at 300 g for 10 minutes at 20°C with the max acceleration and min deceleration settings during the centrifugation. Cells were re-suspended with 10%FBS+RPMI 1640 for assay. Mycoplasma-free Hep3b-hPDL1-OS8 cells were prepared in a 15cm dish with the confluency kept at 60-80% before use. Cells were trypsinized with TrypLE™ Express, (#12605-036, ThermoFisher, MA) and collected to a 50mL centrifuge tube, then centrifuged at 1000 rpm for 5 minutes. The supernatant was discarded, and cells were re-suspended with mitomycin (#H33020786, Zhejiang, China) 10µg/mL in 5-10mL 10%FBS + RPMI 1640 medium. Cells were incubated at 37°C for an hour. After incubation, cells were washed in PBS and cell counting performed with hemocytomete and centrifuged at 1000 rpm for 5 minutes at 20°C with supernatant discarded. Cells were then re-suspended with 10% FBS + RPMI 1640 assay. PBMC stimulation was performed by adding PBMC at 5E4 cell/well with 100μL/well on a 96 well microplate. 50 μL/well of a series of 5X OD1 v2, 5X PD1 WT, or hIgG4 protein solution was added followed by APC Hep3b-hPDL1-OS8 at 5000 cell/well for 50μL/well. The mixed solution was incubated at 37°C for 72 hours and placed in -20°C before ELISA test. The IFN-γ concentration was tested with the ELISA kit according to manufacturer's protocol (#DY285, R&D Systems, MN). The plate was read at 450nm wavelength.